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Meeting Report

Protein Misfolding and Aggregation in Ageing and Disease

Molecular Processes and Therapeutic Perspectives

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Although intensively researched, the fundamental mechanism of protein misfolding that leads to protein aggregation and associated diseases remains somewhat enigmatic. The failure of a protein to correctly fold de novo or to remain correctly folded can have profound consequences on a living system especially when the cellular quality control processes fail to eliminate the rogue proteins. Over 20 different human diseases have now been designated as 'conformational diseases' and include neurodegenerative diseases such as Alzheimer's disease (AD), Huntington's disease (HD) and Creutzfeldt Jakob disease (CJD) that are becoming increasingly prevalent in an ageing human population. Such diseases are usually characterised by the deposition of specific misfolded proteins as amyloid fibrils and hence are often referred to as the amyloidoses.

A recent Jacques Monod Conference entitled "Protein Misfolding and Aggregation in Ageing & Disease" and held in Roscoff (France) in April 2007, brought together 30 leading scientists and a cohort of young scientists actively researching into the amyloidoses and other conformational diseases. The aim of the conference was to provide a comprehensive description, at both the molecular and cellular level, of what we currently know about the early steps of protein aggregation, the high resolution structures of pre fibrillar and fibrillar amyloid states, and our ability to predict the aggregation propensity of polypeptides. A better understanding of these is crucial if we are to be able to rationally design novel therapeutics to prevent these diseases from becoming more prevalent. Together with who also attended the conference, the meeting also set out to identify the future directions this research should take. The following provides a snapshot of some of the exciting new developments reported in Roscoff.

The conference, which received generous support from the French Centre National de la Recherche Scientifique (CNRS), dealt with five main areas: the molecular events in protein aggregation, cellular factors important for protein folding and aggregation, the structure of amyloids, the disease implications of protein aggregation and finally therapeutic approaches to protein misfolding disorders. The meeting was also used by the publishers Landes Bioscience to launch *Prion*.

PROTEIN MISFOLDING: A HISTORICAL PERSPECTIVE

Although the presentations at the conference covered much new ground, a key historical perspective was provided in the opening talk of the conference given appropriately by Michel Goldberg. Goldberg, who was associated with Monod and his work for some 45 years, presented Monod's original ideas on protein folding and aggregation and outlined his own contribution to these seminal studies. Monod became interested in protein folding in the 1960's and was the first to state that protein folding can be viewed as a "second translation of the genetic message". In their seminal work, Goldberg and Monod highlighted the critical role that the experimental conditions, in particular protein concentration and buffer composition, play in correct protein folding in the test tube. Goldberg then went on to propose that the intermolecular interactions leading to the ordered aggregation of a polypeptide is specific to that polypeptide and does not affect the aggregation of another sequence unrelated polypeptide. Thus, Monod and Goldberg's contribution to the field of protein folding was to recognize the problem and to set in motion several of the research themes that formed the basis of this timely Jacques Monod Conference.

THE STRUCTURE OF AMYLOIDS

The diagnostic amyloid fibrils associated with a variety of proteins and different fatal diseases contain a common structural feature, namely a cross-β spine. Louise Serpell

(Sussex) described what we have learnt about the architecture of these fibrils from x-ray fiber diffraction images, electron microscopy and protein crystallography. Using short designed peptides Serpell's group has been able to explore how primary sequence influences the amyloid structure. Using a new computer program Serpell showed how one can simulate the diffraction patterns and the packing of peptides within fibrils and crystals. This has allowed them to assess whether the various models for amyloid structure fit the experimental data.

High resolution NMR approaches have also been applied to the study of a range of other amyloids. Guy Lippens (Lille) showed how NMR and fluorescence spectroscopy have been used to study the role of Tau phosphorylation in its aggregation and interactions with microtubules. Tau binds to microtubules in a given conformation and a number of resonances observed in by NMR for soluble Tau, disappear when the protein is bound to microtubules. This structure based approach has allowed them to dissect the role of individual phosphorylation sites on the structure and function of Tau. Anja Böckman (Lyon) illustrated the input of solid state NMR measurements in determining the conformation of polypeptides in high molecular weight assemblies using the protein Crh from *Bacillus subtilis*. The Crh protein retains its native structure upon precipitation under physiological conditions into amorphous aggregates. Böckman showed that conformational changes can occur within these aggregates upon increase of the temperature. A partially unfolded intermediate state is then reached which converts into β-sheet rich structures.

A full elucidation of the link between the structure and infectivity of amyloid forming proteins is crucial in order to understand the mechanism by which prions, a set of infectious amyloids or amyloid like assemblies, propagate. Using a combination of solution NMR and amide hydrogen/deuterium (H/D) exchange Jonathan Weissman (San Francisco) described the structural basis of yeast prion variants. He showed that two different [PSI$^+$] prion 'variants' show major structural differences specifically due to changes within two amino acid stretches at the N terminus of the underlying Sup35p prion protein. The core of the fibrils formed by the two different strains have different sizes in different prion variants and he proposed that this structural difference could account for the propensity of different prion variants to propagate more or less efficiently. In an effort to define the minimal part is responsible of prion aggregation, Christiane Ritter (Braunschweig) described recent NMR structural studies that have defined the 'infectious fold' of the prion HET s that controls vegetative incompatibility in another fungus, *Podospora anserina*. She proposed that the dimer like character of the arrangement has evolved to facilitate nucleation because fewer monomers are needed to form a stable nucleus as compared to, for example, Aβ.

MOLECULAR EVENTS IN PROTEIN AGGREGATION

The pioneering work of Chris Dobson (Cambridge) has established that any protein, including highly helical polypeptides can take up an amyloid conformation under defined experimental conditions and hence amyloidogenesis is likely to be a generic property of polypeptides. Dobson stressed that misfolding is the default pathway for a newly synthesized protein and thus more 'typical' than its correct folding. Using the lysozyme/clusterin interaction, Dobson showed that protein folding intermediates can be displaced from a productive assembly pathway by interacting with ligands. He also described his group's work on the physical properties of amyloid fibrils using Atomic Force Microscopy (AFM). The amyloid fibrils appear significantly more rigid than other 'rigid' cellular structures such as actin filaments and microtubules and exhibit a slightly higher rigidity than steel wires with a comparable thickness.

To further understand the molecular events at the origin of protein aggregation it is critical to identify the folding intermediates that are on and off the fibrillar assembly pathways and the key steps in the assembly process. One approach was described by Sheena Radford (Leeds) who reviewed her group's in vitro studies on the aggregation of β2 microglobulin (β2m) into ordered, insoluble aggregates. Radford's group have identified aggregation prone sequences within β2m and begun to define the sequence or structural properties that impose the amyloidogenic behaviour played by different sequence determinants. The results strikingly suggest that β2m assembles under physiological conditions into native like fibrils that look like amyloids after populating a set of folding intermediates. At lower pH (2.5) these amyloid fibrils form much faster because a set of unfolding intermediates that are not detected at neutral pH, are more highly populated.

Similarly, Ineke Braakman (Utrecht) described how the endoplasmic reticulum (ER) handles the large increase in folding demand when resting B lymphocytes differentiate into plasma cells that need to correctly fold and rapidly secrete thousands of antibody (IgM) molecules. During such differentiation the IgM accumulates as assembly intermediates in the ER. Using a proteomics approach her group has shown that the levels of all the molecular chaperones resident in the ER increase dramatically during lymphocyte differentiation. One novel putative chaperone is particularly overexpressed and together with the Hsp70 related chaperone BIP, is associated in the ER with the heavy chain of IgM.

The use of novel techniques such as ion mobility mass spectrometry (IMMS) is allowing researchers to better document non covalent interactions during amyloid formation. David Teplow (Los Angeles) described interdisciplinary studies, using in hydro, in vacuo, and in silico approaches, to provide new insights into the differences in the oligomeric states of the two key amyloidogenic peptides associated with AD, namely Aβ40 and Aβ42. For example, in collaboration with Dr. Michael Bowers (Santa Barbara), ion mobility spectroscopy (IMS) techniques revealed that Aβ40 forms largely dimers, trimers, and tetramers, whereas Aβ42 forms additional metastable oligomeric species (i.e., hexamers and dodecamers). IMS coupled with computational techniques will allow in the near future the determination of the geometry of Aβ oligomers that may account for the differences in their assembly properties, differences likely to underlie the enhanced neurotoxic activity of Aβ42.

PREDICTING THE PROPENSITY TO AGGREGATE

The aggregation propensity of a polypeptide can be predicted to some extent by sequence comparison and when taking into account the hydrophobicity of amino acid residues within a sequence. Fabrizio Chiti (Florence) reported his theoretical approach, developing an algorithm that allows them to predict with some accuracy amyloid forming regions in a polypeptide. This has allowed them to identify key regions of the sequence in several amyloidogenic proteins including residues in two amyloidogenic proteins, the mammalian

Tau protein and the yeast prion protein Sup35p. Using a peptide from horse heart apomyoglobin as a model his group have also shown that when a given polypeptide sequence is scrambled the kinetics of assembly change dramatically and this is due to the different distribution of amyloidogenic residues within the sequence.

In silico approaches have also been developed by Frédéric Rousseau (Brussels). Rousseau discussed the differences between amorphous aggregation and amyloidoses. Both TANGO, the widely used aggregation prediction algorithm as well as WALTZ, a new sequence based, amyloid specific prediction algorithm, was described. Using TANGO to screen 28 different proteomes Rousseau found that typically greater than 95% of proteins for a given proteome contain at least one aggregation prone sequence. Rousseau also demonstrated that evolution drives the loss of strongly aggregating sequences and where such sequences remain, in order not to aggregate, proteins have evolved to include charged residues (especially Arg, Pro and Lys) flanking the hydrophobic, aggregation prone residues. Such residues therefore act as 'gatekeepers' that efficiently oppose aggregation and may be recognized by the chaperone machinery and provide a degree of substrate specificity in such recognition. Philippe Derreumaux (Paris) also demonstrated the value of such computer simulations to further increase our understanding of the early steps of polypeptide aggregation and focused on our current understanding of the dynamics and free energy surface of the assembly of amyloid forming peptides that has emerged from coarse grained protein simulations. Alfonso De Simone (Naples) illustrated how replica exchange molecular dynamics can be used to study the structural basis of amyloid fibre formation and stabilization using the amyloid forming peptide GNNQQNY while Riccardo Pellarin (Zurich) described the molecular dynamics simulations of a coarse grained polypeptide model that is able to form fibrils spontaneously. The simulation results depicted the complex relationships between the polypeptide conformational landscape and the nucleation/elongation pathways of amyloid fibrils.

CELLULAR FACTORS IMPORTANT FOR PROTEIN FOLDING AND AGGREGATION

A plethora of molecular chaperones and their co chaperones contribute to correct de novo folding of a polypeptide chain, but also come into play where protein folding goes wrong and the polypeptide chain aggregates. Several speakers at the conference described a range of studies carried out either in vivo or in vitro that illustrated the key role of such cellular factors in both protein folding and aggregation.

Studies of the cellular factors that modulate protein aggregation and/or prion propagation in yeast have been especially informative. Ronald Melki (Gif sur Yvette) described his studies on the roles played by various molecular chaperones in the in vitro assembly of the Gln/Asn rich yeast prion proteins Ure2p and Sup35p. He showed that several families of molecular chaperone modulate the assembly of Ure2p and Sup35p into fibrils in vitro; in particular the Hsp70 and Hsp40 family members sequester the Gln/Asn rich yeast prions into an assembly incompetent state while the chaperone Hsp104 favors nucleation and assembly. Melki proposed that these functional differences among the different chaperones modulate the propagation of the prion traits through a finetuning of the oligomeric state of prion proteins in vivo. Using an in vivo approach Mick Tuite (Canterbury) described what we have learnt about the role played by Hsp104 in the propagation of the prion form of Sup35p. Reversibly inhibiting the ATPase activity of this chaperone using guanidine hydrochloride, has allowed his group to probe the role played by Hsp104 both in the propagation and de novo formation of the [*PSI*$^+$] prion and by applying a complex statistical model to these data, an indirect means of establishing the number of prion seeds ('propagons') necessary for continued propagation of the prion state has been developed.

Further insights into the role of cellular factors in yeast prion propagation were provided by Tricia Serio (Rhode Island) who described her in vivo studies of the dynamics of the aggregation of Sup35p using real time analysis of fluorescently tagged Sup35p. What emerged from her studies is that yeast prions are propagated via three discrete dynamic transitions in the structure of existing Sup35p molecules. The existing soluble Sup35p is almost immediately converted to an insoluble form when a [*PSI*$^+$] cell is mated to a prion free [*psi*$^-$] cell and that this remodeling is independent of Hsp104 and its associated ATPase activity. Serio concluded that the primary role of Hsp104 is in fragmenting Sup35p aggregates, but that in addition it may play a secondary role in nucleation, as was also discussed by Melki.

Yeast based assays have also been used by Ulrich Hartl (Martinsried) to show how polyQ proteotoxicity can be modulated by molecular chaperones. For example, Hsp70 in conjunction with its co chaperones from the Hsp40 family affect polyQ aggregation and suppress its toxicity while the eukaryotic cytoplasmic chaperonin TRiC/CCT acts synergistically with Hsp70 in this process. Interestingly, his studies on htt toxicity in yeast also revealed that overexpression of the chaperone Hsp104 drives the toxic polyQ proteins into larger inclusions with shorter fibrils and reduced toxicity.

Cellular factors other than chaperones that affect protein aggregation and its associated toxicity were described by Cristina Cecchi (Florence). She showed that membrane cholesterol modulates amyloid cytotoxicity and that membranes enriched in cholesterol incorporate smaller amounts of the toxic pre fibrillar Aβ42 oligomers leading to the suggestion that cholesterol may be used to protect against neurodegeneration. Other factors such as kinases and the prolyl cis/trans isomerase Pin1 may also act as modulators of protein aggregation as shown by Luc Buée's (Lille) group for Tau.

CELLULAR MODELS FOR PROTEIN AGGREGATION

An increasingly important tool for those trying to understand the molecular basis of conformational diseases is a tractable yet authentic cellular model. This was illustrated throughout the meeting by various speakers who described their use of yeast (especially *Saccharomyces cerevisiae*), the fruit fly *Drosophila melanogaster*, the worm *C. elegans* and both cultured mammalian cells and transgenic mice. One important new model described by several speakers was the use of fruit fly models to explore the cellular toxicity and pathology linked to amyloids and other protein aggregates. For example, Leila Luheshi (Cambridge) reported how the expression of variants of the human amyloid Aβ40/42 peptide in transgenic flies leads to neuronal alterations that in turn affect the mobility and longevity of the fly. In validating it as a disease model, Luheshi was able to show a good correlation between the propensity of the amyloid Aβ to form protofibrils and toxicity although there was much less of a correlation between toxicity and the propensity to form fibril mature fibrils. Christelle Lasbleiz (Paris) also showed that the fruit fly can be

used as a conditional model of spinocereballar ataxia 7 (SCA7) while David Lomas (Cambridge, UK) used a fruit fly model to show that oxidative stress underlies toxicity of amyloid Aβ aggregation while ferritin protects against toxicity in this model.

Cellular models for studying Htt/polyQ aggregation were also described by several groups including Anne Bertolotti (Cambridge) and Ron Kopito (Stanford). Bertolotti used a combination of cultured mammalian and yeast cells to probe the importance of sequence features and cellular factors that are important for *htt* aggregation and associated toxicity. Kopito reported the recent exciting finding that exogenously supplied polyQ aggregates can be taken up by cultured mammalian cells and then propagated by a prion like mechanism, recruiting soluble cytoplasmic proteins providing they share homologous amyloidogenic sequences. This recruitment leads to a change in heritable phenotype and the property transmitted to daughter cells during mitosis. This latter finding raises the intriguing possibility that the PrP prion protein is not the only type of 'infectious amyloid' in mammals.

WHAT MAKES A PROTEIN AGGREGATES TOXIC?

The formation and propagation of misfolded forms of certain cellular proteins underlie many of the 'conformational diseases' discussed at the meeting. In some cases these forms are toxic—as in the case of the various amyloidoses while in other cases e.g. Gaucher disease, a common lysosomal storage disease the disease pathology is associated with a loss of function of the protein in question rather than a gain of toxicity. The deposition of proteins of aberrant conformation is the hallmark of several neurodegenerative diseases such as AD, HD and prion diseases such as CJD, but it remains unclear as to whether the soluble oligomers that form during the early stages of assembly or the large insoluble often fibrillar assemblies that are most toxic to the cells. Using htt a polypeptide carrying polyQ expansions involved in HD disease, Philippe Djian (Paris) reported that the most toxic htt aggregates appear to be those of small size that are located in the nucleus.

THE POTENTIAL FOR THERAPEUTIC INTERVENTION

Blocking the formation of the disease associated protein aggregates represents a major target for therapeutic intervention and as was evident from several presentations at the conference, real progress is beginning to be made in the search for small molecule inhibitors—be they natural or synthetic in origin—which block the formation of toxic aggregates or which abrogate their toxicity.

One of the challenges is to develop a suitable assay for candidate compounds that has sufficiently high throughput to allow the screening of the ever-increasing chemical libraries. Erich Wanker (Berlin) and his colleagues have used a cell free screen to identify a number of small molecules that inhibit polyQ/htt aggregation. The ability of these compounds to reduce fibril formation and suppress toxicity at the organismal level was confirmed using yeast, Drosophila and mammalian cell based assays. Intriguingly some of the compounds examined promoted the formation of off pathway non toxic oligomers rather than blocking protein aggregation per se. Among such compounds identified was epigallocatechin gallate (EGCG) a component of green tea and the htt oligomers formed in the presence of EGCG are not toxic to cultured cells. Inducing the conversion of amyloidogenic proteins into off pathway and non toxic oligomeric structures may therefore represent a promising new therapeutic strategy to prevent or at least slow down the pathogenesis associated with amyloidoses.

While the actual target for EGCG and other compounds remains to be defined, Marc Blondel (Brest) described the mechanism of action of a group of antiprion drugs originally identified by his group using both yeast and mammalian cell based assays. The cellular target of two of the most potent antiprion compounds they discovered, 6 aminophenanthridine and another drug already in the clinic for other applications, appear to target a chaperone activity associated with the heavy subunit of the ribosome (50S). Both compounds eliminate fungal and mammalian prions providing strong evidence for the universality of the process by which prions are propagated in fungi and humans.

Several other approaches to screen for new therapeutic agents active against one or more conformational diseases were also described. Based on the observation that the peptide NFGAIL loses its assembly propensity when the residue F is changed to Y or aliphatic amino acids, but not W, Ehud Gazit (Israel) suggested that the packing of aromatic residues is critical for the initiation of assembly and therefore should be targeted for anti assembly therapeutic strategies. Based on these findings, a novel inhibitor was found to restore cognitive performance in a mouse AD model. Similarly, and following the observation that a peptide derived from IAPP (IAPP GI) inhibits the cytotoxic self assembly of both Aβ and IAPP polypeptides, Aphrodite Kapurniotu (Aachen) suggested that such synthetic peptides may constitute promising therapeutic strategies targeting both AD and type 2 diabetes.

Jeff Kelly (La Jolla) proposed a novel way of treating type 1 Gaucher disease that is associated with the loss of function of a glucocerebrosidase. Kelly's approach was to identify inhibitors of this enzyme that would stabilize a significant proportion of the enzyme molecules leading to the accumulation of the enzyme inhibitor complex within the cells. At very high concentrations of the enzyme inhibitor complex, a fraction of the enzyme is found unbound to its inhibitor because of the dissociation constant and this leads to a basal level of enzymatic activity and hence survival.

In addition to identifying compounds, cellular targets are also being identified to facilitate rational drug design strategies. Paul Muchowski (San Francisco) described his group's use of a yeast based screen to identify genes associated with the toxicity of a mutant fragment of htt and this has led to the finding that deletion of the yeast gene *BNA4*, that encodes kynurenine 3 mono oxygenase (KMO), alleviates such toxicity. KMO is a mitochondrial enzyme implicated in the kynureinine pathway of tryptophan degradation and is also found exclusively in the microglia in the central nervous system (CNS). Muchowski suggested that the mutant htt may induce a transcriptional defect that activates the kynureinine pathway in the CNS and that this contributes to the neurodegeneration seen in HD patients. Human KMO therefore represents a novel 'druggable' target since its inhibition, as already shown by Muchowski's group, has significant beneficial effects in a mouse model of HD.

WHAT DOES THE FUTURE HOLD?

A lasting impression from this timely conference was that a conceptual framework is now beginning to emerge that will allow

us to better understand the way in which the unique folds found in proteins are attained within a common mechanism of protein folding. One of the greatest challenges we now face is to fully understand how the misfolding and/or misassembly of certain intracellular proteins leads to the development of 'gain of toxic function' diseases such as HD. Evidence presented by a number of speakers suggests that soluble amyloid like oligomers or β sheet rich protofibrils are the major toxic species behind these diseases rather than the diagnostic mature fibrils or amyloid plaques. The identities of the toxic aggregates and the mechanisms by which they induce dysfunction and toxicity remain the subject of intensive debate. The establishment and validation of a range of cellular models for conformational diseases, including transgenic animals, yeast and the fruit fly, will soon allow for obtaining this crucial information on the nature of the toxic oligomers, cellular factors that modulate their toxicity and the identification of viable therapeutic strategies to override the proteotoxicity. The application of in silico approaches to these problems is also beginning to bear fruit.